



APPENDIX I

Detection of Disseminated Colorectal Cancer Cells in Lymph Nodes, Blood and Bone Marrow

Jürgen Weitz, Peter Kienle, Achim Magener, Moritz Koch, Andrea Schrödel, Frank Willeke, Frank Autschbach, Jeannine Lacroix, Thomas Lehnert, Christian Herfarth, and Magnus von Knebel Doeberitz¹

Division for Molecular Diagnostics and Therapy, Department of Surgery [J. W., P. K., M. K., A. S., F. W., J. L., M. v. K. D.]; Division of Surgical Oncology, Department of Surgery [T. L., C. H.]; and Department of Pathology [A. M., F. A.], University of Heidelberg, D-69120 Heidelberg, Germany

ABSTRACT

Tumor progression after curative resection of colorectal cancer is caused by tumor cell dissemination, currently undetected by standard clinical staging techniques. The detection of disseminated tumor cells could help to identify a patient subgroup at risk for disease relapse who could benefit from adjuvant therapy. In addition, the significance of lymphogenic compared with hematogenic colorectal cancer cell dissemination is unknown. However, this knowledge would strongly influence the development of future therapeutic regimes. The purpose of this study was to determine the extent of colorectal cancer cell dissemination in lymph nodes compared with blood and bone marrow. Using a CK 20-reverse transcription (RT)-PCR assay, we examined 279 lymph nodes, blood, and bone marrow samples from 20 patients with colorectal cancer. Of 16 patients (11 patients stage I, 5 patients stage II) with histopathologically tumor-free lymph nodes: 14 patients (10 patients stage I, 4 patients stage II) were found to have tumor cells in paracolon lymph nodes; 12 patients (8 patients stage I, 4 patients stage II) were found to have tumor cells in the lymph nodes along the mesentery vessels; and, remarkably, 6 patients (4 patients stage I, 2 patients stage II) were found to have tumor cells in the apical lymph nodes. In contrast, tumor cells were detected in only two blood and three bone marrow samples of these patients. Thus, lymphogenic tumor cell dissemination is a very common and early event in colorectal cancer, preceding hematogenic tumor cell dissemination. In addition, our data strongly suggest that the detection of tumor

cells in the apical lymph node by CK 20-RT-PCR has prognostic relevance. Our results underline the therapeutic importance of meticulous lymph node dissection and demonstrate that the detection of lymphogenic or hematogenic tumor cell dissemination by CK 20-RT-PCR will significantly improve current tumor staging protocols.

INTRODUCTION

Patients with colorectal cancer initially presenting with resectable tumors and tumor-free lymph nodes (UICC² stage I or II) are generally considered as patients at low risk for recurrence. Therefore, adjuvant therapy is not recommended in these cases. Despite the low tumor stage, about 30–40% of these patients subsequently develop recurrent disease (1). These findings indicate that hematogenic or lymphogenic dissemination of metastatic tumor cells, not detectable by conventional staging techniques, must have occurred in some of these patients. Detection of disseminated tumor cells in patients with colorectal cancer UICC stage I or II may identify high-risk patients for tumor recurrence, who could benefit from adjuvant therapy regimens. The comparative analysis of lymph nodes, blood, and bone marrow may define the relative incidence of hematogenic *versus* lymphogenic tumor cell dissemination in colorectal cancer. This data could help to optimize strategies for the treatment of colorectal cancer, for example, by defining the relative importance of surgical lymphadenectomy *versus* systemic adjuvant therapy.

Immunocytological methods have been applied to detect hematogenic tumor cell dissemination (2). Tumor cell detection was clearly related to an earlier relapse and decreased survival of the respective patients. RT-PCR-based protocols have further improved the sensitivity and specificity of detection systems for disseminated cancer cells in blood and bone marrow (3–9).

The second route of tumor cell dissemination is via the lymphatic system (10). Because the routine histopathological methods of lymph node examination show limited sensitivity, several techniques have been studied in an attempt to increase the detection of occult lymph node micrometastases, including serial sectioning of lymph nodes (11), immunohistology (12–17), and, finally, PCR-based techniques (18–26). These studies, however, revealed contradictory results regarding the incidence of occult lymph node micrometastases and their prognostic significance.

Thus, the extent and prognostic significance of lymphogenic tumor cell dissemination in colorectal cancer has still not been sufficiently defined. In addition, the relative significance of lymphogenic *versus* hematogenic tumor cell dissemination is

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¹ To whom requests for reprints should be addressed, at Division for Molecular Diagnostics and Therapy, Department of Surgery, University of Heidelberg, INF 110, D-69120 Heidelberg, Germany. Phone: 49-6221-56-2876; Fax: 49-6221-56-5981; E-mail: knebel@med.uni-heidelberg.de.

² The abbreviations used are: UICC, International Union Against Cancer; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde phosphate dehydrogenase; CEA, carcinoembryonic antigen.

still a matter of debate (27). The purpose of the present study was to determine the extent of dissemination of colorectal cancer cells in lymph nodes and to correlate these results with the detection of disseminated tumor cells in bone marrow and blood samples of the same patients.

PATIENTS AND METHODS

Lymph Node and Tumor Sampling. Lymph nodes were freshly harvested from the resected specimen. Initially, the mesentery was separated from the gut to prevent any cross-contamination with CK 20 positive cells. Then the lymph nodes were dissected from the mesentery. The location of the lymph nodes was recorded [apical lymph node, lymph node at the central lymphovascular ligation, lymph nodes at the main vessel (for example, inferior mesenteric artery), and paracolon lymph nodes]. The lymph nodes were then halved; one-half was used for conventional histopathology, and the other half was shock-frozen in liquid nitrogen and stored at -70°C . From the fresh resection specimen, a tumor sample was excised and shock-frozen. Before RNA extraction, frozen tissue sections of tumors and lymph nodes were performed. From each lymph node half, 20 representative sections with a diameter of 20 μm were used for RNA extraction.

Blood and Bone Marrow Samples. After the induction of general anesthesia, 10-ml blood samples were obtained preoperatively through a central venous catheter in the vena cava superior and were diluted with 10 ml of PBS. Bone marrow samples (10-ml) were obtained concurrently by aspiration from both iliac crests. After density centrifugation through Ficoll-Paque (Pharmacia; 30 min, $400 \times g$) mononuclear cells were harvested from the interphase and washed twice in PBS. The cell pellet was then shock-frozen in liquid nitrogen and stored at -70°C .

RNA Extraction. RNA extraction from peripheral mononuclear blood cells and from bone marrow samples and frozen tissue sections of tumors and lymph nodes was performed as described previously (9).

RT-PCR. CK 20-RT-PCR was performed as described previously (9). PCR products were analyzed by electrophoresis on 2% agarose gels. CK 20-PCR products were blotted onto nylon membranes (Hybond N+, Amersham Life Science, Buckinghamshire, United Kingdom) and hybridized with a chemoluminescence-labeled oligonucleotide probe (ECL detection system, Amersham Life Science) comprising nucleotides 5269–5280 on exon 1 and nucleotides 7429–7448 on exon 2 of the CK 20 sequence (CTG CGA AGT CAG ATT AAG GAT G) as recommended by the supplier.

RNA quality and performance of reverse transcription of all of the analyzed samples was confirmed by RT-PCR amplification of GAPDH transcripts as described previously (28).

Patients. Informed consent was obtained from all of the patients. The study protocol was approved by the Ethics Committee of the University of Heidelberg.

Twenty patients treated at the Department of Surgery, University of Heidelberg (13 male, 7 female; ages 48–86, mean 66.4) were included. All of the patients had histologically confirmed colorectal adenocarcinoma (9 rectal carcinoma, 11 colon carcinoma) undergoing curative (R0) resection according to the

“no-touch isolation” technique (29) with systematic lymph node dissection. Tumor stage and grading were classified according to the 5th edition of the TNM classification of the UICC (30).

Paracolon lymph nodes from 22 patients undergoing colorectal resection for benign diseases (sigmoid diverticulosis, Crohn’s disease and ulcerative colitis) served as controls.

RESULTS

Specificity of CK 20-RT-PCR

Paracolon lymph node samples ($n = 22$) from 22 patients undergoing colorectal resection for benign disease were examined for expression of CK 20. None of these samples revealed a detectable CK 20-RT-PCR amplification product. All of the lymph nodes showed a positive GAPDH amplification signal, which confirmed the quality of the RNA and the adequate performance of reverse transcription. The 20 analyzed primary tumor samples all tested positive for CK 20 mRNA expression. Specificity of CK 20-RT-PCR for tumor cell detection in blood and bone marrow has been demonstrated previously (7, 9).

Patient Study

Detection of Disseminated Tumor Cells in Lymph Node Samples of Patients with Colorectal Carcinoma. In a prospective study, we analyzed 279 lymph nodes from 20 patients with colorectal cancer (mean, 13.95 lymph nodes per patient) histopathologically and by CK 20-RT-PCR. Of the 279 examined lymph nodes, 10 were positive by histopathological examination; all of these also revealed a positive CK 20 signal. Using the RT-PCR, 137 (50.9%) of the 269 histopathologically tumor-free lymph nodes revealed CK 20 amplification products.

Four of the 20 patients had positive lymph nodes on routine histopathological examination (stage III), and 16 patients had negative lymph nodes (11 patients stage I, 5 patients stage II). CK 20-RT-PCR revealed one or more positive lymph nodes in 14 of the 16 patients [UICC stage I or II (10 of 11 patients stage I; 4 of 5 patients stage II; Table 1; Fig. 1]. The results were stratified according to lymph node location [apical lymph node, lymph nodes along the main vessel (for example, inferior mesenteric artery), and paracolon lymph nodes]. In the patients with UICC stage I or II tumors, the apical lymph node revealed a positive CK 20 signal in 6 (37.5%) of 16 patients (4 of 11 patients stage I, 2 of 5 patients stage II), the lymph nodes at the main vessel in 12 (75%) of 16 patients (8 of 11 patients stage I, 4 of 5 patients stage II), and the paracolon lymph nodes in 14 (87.5%) of 16 patients (10 of 11 patients stage I, 4 of 5 patients stage II; Tables 1 and 2). All of the six patients with positive apical lymph node also revealed positive lymph nodes along the main vessel and in the paracolon tissue. All of the six patients with a negative central lymph node but positive lymph nodes along the main vessel also had positive paracolon lymph nodes.

Comparative Analysis of Tumor Detection Rates in Lymph Nodes, Venous Blood, and Bone Marrow Samples. Bone marrow samples from 16 patients and preoperative blood samples from 20 patients were analyzed in addition to the lymph nodes. In patients at UICC stage I or II, 3 (21.4%) of 14 bone marrow samples and 2 (12.5%) of 16 blood samples revealed a CK 20 amplification product (Table 2).

Table 1 Results of the detection of tumor cells in lymph nodes by conventional histopathology compared to CK 20-RT-PCR

Patient No	Stage	Conventional histopathology	CK 20-RT-PCR	Location of CK 20-positive nodes		
				Central	Main vessel	Paracolic
1	I	0 of 14	7 of 14	Pos ^a	1 of 4	5 of 9
2	II	0 of 10	0 of 10	Neg	0 of 1	0 of 8
3	I	0 of 13	4 of 13	Neg	0 of 7	4 of 5
4	I	0 of 7	6 of 7	Pos	1 of 2	4 of 4
5	I	0 of 11	4 of 11	Neg	2 of 2	2 of 8
6	I	0 of 12	2 of 12	Neg	0 of 4	2 of 7
7	I	0 of 13	0 of 13	Neg	0 of 4	0 of 8
8	II	0 of 14	9 of 14	Neg	1 of 3	8 of 10
9	II	0 of 15	3 of 15	Neg	1 of 6	2 of 8
10	I	0 of 17	10 of 17	Neg	2 of 4	8 of 12
11	I	0 of 10	4 of 10	Neg	2 of 2	2 of 7
12	I	0 of 19	19 of 19	Pos	11 of 11	7 of 7
13	I	0 of 15	13 of 15	Pos	6 of 6	6 of 8
14	II	0 of 23	16 of 23	Pos	5 of 6	10 of 16
15	II	0 of 24	13 of 24	Pos	3 of 9	9 of 14
16	I	0 of 18	6 of 18	Neg	3 of 9	3 of 8
17	III	3 of 11	7 of 11	Neg	4 of 7	3 of 3
18	III	1 of 9	6 of 9	Neg	5 of 6	1 of 2
19	III	3 of 13	12 of 13	Pos	5 of 6	6 of 6
20	III	3 of 11	6 of 11	Pos	2 of 4	3 of 6
		10 of 279 3.58%	147 of 279 52.7%	8 of 20 40%	54 of 103 52.4%	85 of 156 54.5%

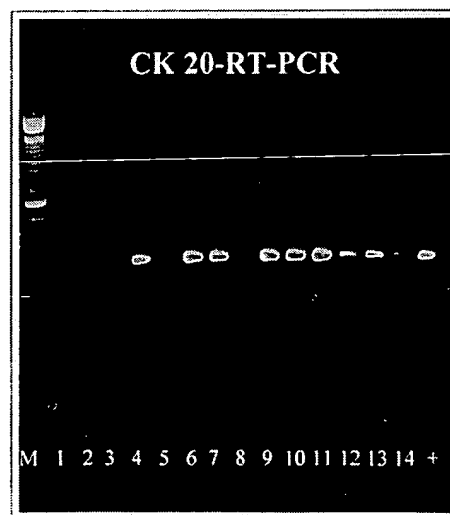
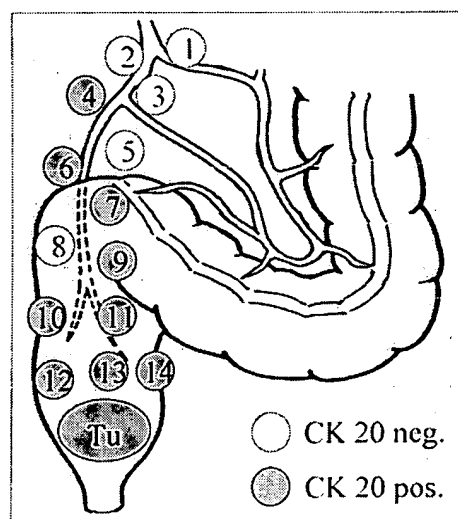
^a Pos, positive; Neg, negative.

Fig. 1 Results from a patient with rectal carcinoma pT₃ pN₀. Left, surgical specimen with the location of tumor and lymph nodes; right, CK 20-RT-PCR amplification products of resected lymph nodes. Tu, tumor; M, molecular weight marker; +, positive control (tumor).

DISCUSSION

Although most patients with colorectal cancer are potentially curable by surgery at presentation, one-half of them will eventually die of the disease. This tumor progression could result from isolated disseminated tumor cells—in lymph nodes, blood, or bone marrow—that are not detected by current staging methods. The objective of adjuvant therapy is to eradicate viable disseminated tumor cells, thereby decreasing disease relapse and

improving patient survival (31). Candidates for postoperative adjuvant therapy are patients at high risk for disease relapse, as judged by current clinical and pathological staging. In the group of patients without distant metastases, lymph node metastases are the most important prognostic factor (10). Consequently, adjuvant chemotherapy is recommended for patients with positive lymph nodes (UICC stage III; Ref. 32). For patients with colon cancer UICC stage I or II, adjuvant chemotherapy did not

Table 2 Results of the comparative analysis of lymph nodes, blood, and bone marrow samples in patients UICC stage I and II by CK 20-RT-PCR

Patient No	Stage	CK 20-RT-PCR	Location of CK 20-positive nodes				
			Central	Main vessel	Paracolonial	Blood	Bone marrow
1	I	Pos ^a	Pos	Pos	Pos	Neg	n.o.
2	II	Neg	Neg	Neg	Neg	Neg	Neg
3	I	Pos	Neg	Neg	Pos	Neg	n.o.
4	I	Pos	Pos	Pos	Pos	Neg	Neg
5	I	Pos	Neg	Pos	Pos	Pos	Pos
6	I	Pos	Neg	Neg	Pos	Neg	Neg
7	I	Neg	Neg	Neg	Neg	Neg	Pos
8	II	Pos	Neg	Pos	Pos	Neg	Neg
9	II	Pos	Neg	Pos	Pos	Neg	Neg
10	I	Pos	Neg	Pos	Pos	Neg	Neg
11	I	Pos	Neg	Pos	Pos	Neg	Neg
12	I	Pos	Pos	Pos	Pos	Neg	Neg
13	I	Pos	Pos	Pos	Pos	Neg	Neg
14	II	Pos	Pos	Pos	Pos	Neg	Pos
15	II	Pos	Pos	Pos	Pos	Neg	Neg
16	I	Pos	Neg	Pos	Pos	Pos	Neg
		14 of 16 87.5%	6 of 16 37.5%	12 of 16 75%	14 of 16 87.5%	2 of 16 12.5%	3 of 14 21.4%

^a Pos, positive; Neg, negative; n.o., not obtained.

achieve a survival benefit, and, thus, adjuvant therapy is not recommended in these stages (32). Although considered at low risk, 30–40% of patients with colorectal cancer UICC stage I and II ultimately develop recurrent disease (1). It is in this patient group that prognostic markers may identify a patient subgroup at high risk for disease relapse who may also benefit from adjuvant therapy, especially because antitumor agents with a low systemic toxicity (for example, monoclonal antibodies) that have also been proven to be effective against dormant tumor cells are now available (33). Because tumor relapse is presumably caused by hematogenic or lymphogenic disseminated tumor cells, the detection of these disseminated tumor cells may identify patients who could benefit from adjuvant therapy.

Focusing on hematogenic tumor cell dissemination, Lindemann *et al.* (2), using immunocytological methods, detected disseminated colorectal cancer cells in bone marrow aspirates in 32% of patients. Tumor cell detection was clearly related to an earlier relapse and decreased survival of the respective patients. RT-PCR-based protocols have further improved the sensitivity and specificity of detection systems for disseminated cancer cells, allowing the identification of approximately one neoplastic cell in 10^7 normal peripheral mononuclear blood cells (3). CK 20 transcripts appear to be good targets for the detection of disseminated colorectal cancer cells because they are expressed in gastrointestinal epithelia, urothelium, or Merkel cells and in their respective tumors but not in other nontransformed tissues (4, 5). Burchill *et al.* (6) described a CK 20-RT-PCR system for the detection of malignant colonic epithelia in blood and bone marrow samples. Soeth *et al.* (7, 8) used a CK 20-RT-PCR assay to detect disseminated cancer cells in bone marrow aspirates and blood samples of gastrointestinal cancer patients. Using a modified CK 20-RT-PCR assay, we were able to detect circulating

tumor cells in 24 of 58 patients with colorectal cancer in correlation to the tumor stage and in 6 of 7 patients who underwent hemihepatectomy for liver metastasis of colorectal cancer (9). We were also able to demonstrate significant intraoperative tumor-cell dissemination in these patients (9).

Because lymph node involvement has proven to be one of the most important prognostic factors for colon cancer patients (10), lymphogenic dissemination of tumor cells is likely to be at least as important as hematogenic cancer cell spread. The routine histopathological methods of lymph node examination show limited sensitivity because clinical pathologists investigate only one or two sections of each lymph node. In addition, the detection of small clusters of tumor cells is difficult. In about one-fifth of the cases primarily diagnosed as lymph-node negative, metastasis can be detected on reexamination by serial sectioning of the lymph nodes (11). Goldenberg *et al.* (12) introduced immunohistochemistry for the detection of tumor cells in lymph nodes that were previously considered free of disease by the standard H&E staining technique. Using immunohistochemistry, tumor cells were detected in about 3.6–6.1% of histopathologically negative lymph nodes (13–16). Of the reexamined patients, 25–36% had to be restaged from UICC stage I-II to UICC stage III. Using the combination of immunohistochemistry and the fat clearance technique, which allows the detection of more lymph nodes in a surgical specimen, even 55% of the reexamined patients had to be restaged upward (17). Because of the contradictory results in the published studies, however, the prognostic significance of immunohistochemically detected lymph node micrometastases remains controversial (13–16).

In an effort to further increase the sensitivity and specificity of tumor cell detection in lymph nodes, genetic methods

were applied. These methods have the advantage of a higher sensitivity and the possibility of examining not only single sections but multiple sections of a lymph node in a single examination. Using the mutant allele-specific amplification method, Hayashi *et al.* (18) were able to detect tumor cells of gastrointestinal carcinomas with mutant *k-ras* or *p53* genes in lymph nodes. However, this method is restricted to tumors with known *k-ras* or *p53* mutations. In addition, positive PCR results may originate from fragments of free tumor DNA rather than from viable tumor cells (19).

Mori *et al.* (20) and Liefers *et al.* (21) used CEA transcripts as targets for detection of micrometastasis in lymph nodes. However, the sensitivity of the used assay had to be reduced to avoid false-positive results. In the study performed by Liefers *et al.* (21), the detection of disseminated tumor cells was associated with an unfavorable prognosis, which suggests that micro-involvement of local lymph nodes is indeed associated with increased relapse rates. However, several arguments have been raised against this study: (a) only seven lymph nodes per patient were investigated; (b) too few negative control samples were included (34); and (c) the sensitivity of the CEA-RT-PCR assay has not been appropriately addressed (35). In addition, concerns about the specificity of the marker (CEA) used for the detection of the cancer cells have again been put forward (36) because CEA transcripts are also detectable in normal lymph nodes of patients who are not suffering from colorectal cancer (37).

Other investigators used RT-PCR amplification of CK 20 (22, 23), matrilysin (24), or CD 44 (25) mRNAs for the same purpose. Tumor cells were detected in lymph nodes of 16–57% of patients with histopathologically negative lymph nodes. Recently, Futamura *et al.* (26) reported the detection of micrometastases by the combination of CEA-RT-PCR and CK 20-RT-PCR in the lymph nodes of 13 of 13 examined colorectal cancer patients at UICC stage I or II. Because only 30–40% of patients with colorectal cancer UICC stage I and II develop recurrent disease, this high detection rate raises doubts concerning the prognostic relevance of these findings.

In this study, we investigated the rate of tumor cell dissemination in the lymph nodes, blood, and bone marrow of 20 patients with colorectal carcinomas using a CK 20-RT-PCR assay.

The sensitivity of the system was evaluated in previous studies (9) and reproducibly allowed the detection of approximately 10 tumor cells in 10 ml of blood. Specificity is a major concern in RT-PCR systems used for the detection of disseminated tumor cells (3). CEA-RT-PCR, for example, is known to yield false-positive results in normal lymph nodes (20, 21, 26). Specificity of the used CK 20-RT-PCR was ensured by the measures discussed previously (9). Lymph nodes of 22 healthy patients undergoing surgery for nonneoplastic colorectal disease consistently tested negative with the established CK 20-RT-PCR. These results demonstrate that normal lymph nodes do not contain CK 20-expressing cells. Even in patients with inflammatory bowel disease, nonneoplastic colon cells do not reach the first local lymph node station. Thus, false-positive results due to the migration of normal colon epithelia are unlikely. There is also concern that DNA-based methods for tumor cell detection may detect free tumor DNA rather than viable tumor cells (19), thereby reducing the specificity of the method. Because free

mRNA is extremely unstable, tumor cells detected by RT-PCR are highly likely to be viable.

Isolated disseminated tumor cells were detected by CK 20-RT-PCR in lymph nodes in 14 of 16 patients UICC stage I and II. In previously published RT-PCR-studies, tumor cells were detected in lymph nodes of 16–57% of tested patients (20–25). Given the higher sensitivity of RT-PCR in relation to immunohistochemistry, these published detection rates are surprisingly low compared with the immunohistochemical data revealing positive lymph nodes in 25–55% of patients. This could be due to the low sensitivity of the hitherto tested RT-PCR assays (1 tumor cell in 1×10^4 to 5×10^5 peripheral mononuclear cells) because single and nonnested PCR-systems were used (22–25). The higher detection rate in our study is presumably the result of the higher sensitivity (1 tumor cell in 10^7 peripheral mononuclear cells) of the nested CK 20-RT-PCR. Using a combination of a nested CK 20-RT-PCR and CEA-RT-PCR, Futamura *et al.* (26) recently reported tumor cell detection in lymph nodes of all of the 13 examined patients UICC stage I or II.

In our study, tumor cells were detected in the lymph nodes of 87.5% of patients UICC stage I and II, and in the study of Futamura *et al.* (26) they were detected in 100% of patients UICC stage I and II. These detection rates are much higher than the known tumor recurrence rate in these stages. Thus, the detection of tumor cells in surgically removed lymph nodes by CK 20-RT-PCR cannot be a prognostic marker *per se*. Because it is well known that the prognosis of patients with histopathologically tumor-infiltrated apical lymph nodes is worse than the prognosis of patients with negative apical lymph nodes (38–44), we stratified the results of the 16 patients UICC stage I and II according to lymph node position: (a) in 8 (50%) of the 16 patients, tumor cells were detected only in lymph nodes along the main vessel and paracolonial lymph nodes without infiltration of the apical lymph node; (b) in 2 (12.5%) of the 16 patients, tumor cells were detected only in paracolonial lymph nodes. Because all of these lymph nodes were surgically removed by lymphadenectomy, they may be of no further prognostic significance; (c) in 6 (37.5%) of the 16 patients, however, tumor cells were also detected in the apical lymph node. Because 30–40% of patients UICC stage I and II will develop recurrent disease, it could be hypothesized that the detection of tumor cells in the apical lymph node by CK 20-RT-PCR indicates tumor cell dissemination beyond the margin of surgical lymphadenectomy. These patients may have a worse prognosis and could benefit from adjuvant therapeutic regimes. This hypothesis remains to be confirmed by a follow-up of our patient cohort.

The evaluation of the relative importance of lymphogenic versus hematogenic tumor cell dissemination in colorectal cancer could provide important data for additional therapeutic strategies. For example, extended surgical lymphadenectomy could be useless in the presence of multiple tumor cells in blood and bone marrow. Clinical studies that address the relative importance of lymphogenic versus systemic tumor dissemination, however, are rare (27). We compared lymphogenic and hematogenic tumor cell dissemination in patients with colorectal cancer by examining lymph nodes, blood, and bone marrow from each patient. Tumor cells were only detected in 12.5% of blood and

21.4% of bone marrow samples from patients UICC stage I or II. Thus, lymphogenic tumor cell dissemination seems to be much more common than detectable hematogenic tumor cell spread in early colorectal cancer. These results are strong arguments for the importance of meticulous lymph node dissection, especially in patients with colorectal cancer in early tumor stages without systemic hematogenic micrometastasis. Adequate surgical lymphadenectomy may, therefore, be the most important factor to prevent recurrent disease in early colorectal cancer by resection of all of the potentially involved lymphatic tissue with the elimination of all of the lymphogenic disseminated tumor cells.

We conclude that the CK 20-RT-PCR is a sensitive and specific tool for the detection of lymphogenic and hematogenic dissemination of colorectal carcinoma. The long-term follow-up of our patients will provide data on the prognostic relevance of isolated disseminated tumor cells in the lymph nodes, blood, and bone marrow of patients with colorectal cancer and may lead to new concepts for adjuvant therapy.

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